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MCF7 breast carcinoma cells were rendered susceptible to infection with recombinant ecotropic retroviruses, through transfection with the gene for murine ecotropic receptor and by testing individual transfectants for infectability. A genetic suppressor element (GSE), derived from the BCL2 gene which inhibits active cell death (apoptosis), was introduced into MCF7 cells. The BCL2-derived GSE sensitized MCF7 cells to different chemotherapeutic drugs, indicating that BCL2 is an important determinant of chemoresistance of these cells. A retroviral library of normalized cDNA fragments, derived from HeLa cell line, was introduced into MCF7 cells, and selection of GSEs that render MCF7 cells resistant to doxorubicin was initiated. A normalized cDNA library is also being prepared from MCF7 cells, for use as a source of GSEs conferring resistance to tamoxifen.

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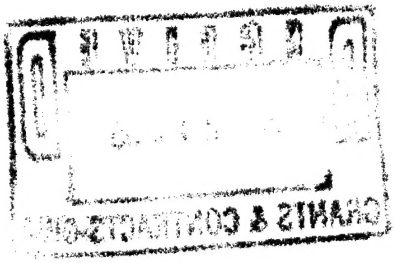

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Introduction

Chemotherapy, based on cytotoxic drugs or hormone antagonists, is widely used as either adjuvant or primary treatment at all stages of breast cancer (1). The drug regimens that are most commonly used for breast cancer include a combination of cyclophosphamide, methotrexate and 5-fluorouracyl (CMF), or doxorubicin (presently the most powerful drug in this group) which is most often used as a single agent in the second line of treatment. In addition, taxol was shown by recent studies to produce significant objective responses in breast cancer. Aside from these chemotherapeutic compounds that are used in different types of cancer, another, breast cancer-specific class of drugs is used in the treatment of estrogen receptor-positive tumors. These agents are antiestrogens, the most widely used of which is tamoxifen (1). The objective of the present grant is to identify genes that determine the sensitivity of human breast carcinoma cells to agents used in breast cancer treatment, including antiestrogens (tamoxifen) and cytotoxic drugs (doxorubicin, cyclophosphamide, methotrexate, 5-fluorouracyl, taxol).

Our approach to the identification of chemotherapeutic sensitivity genes is based on the isolation of genetic suppressor elements (GSEs), derived from such genes and inducing cellular resistance or sensitivity to the corresponding agents. GSEs are short cDNA fragments that counteract the genes from which they are derived by encoding inhibitory peptides or antisense RNAs (2). We have previously developed the methodology for GSE selection from retroviral libraries carrying short random fragments of normalized (uniform-abundance) cDNA from mammalian cells (3,4). Using this approach, we identified several GSEs conferring resistance to anticancer drugs or inducing neoplastic transformation (4-6). The same strategy is being used in the present project to identify GSEs that render breast carcinoma cells resistant or sensitive to chemotherapeutic agents. In one arm of the project, GSEs inducing resistance to a metabolite of tamoxifen will be cloned by expression selection in MCF7 cells. The cloned GSEs will be used to isolate full-length cDNA sequences of the corresponding genes, and the effects of individual GSEs on hormone responsiveness and drug resistance will be investigated. In another arm of the project, different GSEs inducing resistance or sensitivity to chemotherapeutic drugs in various cell types, will be tested for their ability to make MCF7 cells resistant to cytotoxic drugs and antiestrogens. The genes giving rise to such GSEs would constitute likely determinants of chemotherapeutic sensitivity in breast carcinoma.

Body

1. Development of a recipient MCF7/ETR cell line

The most efficient of the protocols used in our laboratory for gene transfer into mammalian cells is retroviral transduction, using 293-derived BOSC 23 packaging cells (7). These cells, however, produce ecotropic virus, which would normally infect only mouse or rat cells that express the

ecotropic receptor (ETR) on their surface. To render MCF7 human breast carcinoma cells susceptible to infection with ecotropic retroviruses, we co-transfected these cells (obtained from ATCC) with pJET2 plasmid carrying the murine ETR gene (8), and D3PVBleo plasmid, carrying a phleomycin resistance gene, using a standard transfection protocol based on calcium phosphate coprecipitation. After three weeks of selection with 20-70 μg/ml phleomycin, about 80 transfectant clones were obtained. 43 of these clones were individually tested for susceptibility to infection with retroviral vector LNCX (9), carrying the *neo* (G418) resistance gene, and generated by transient transfection into BOSC 23 ecotropic packaging cells. The infectability of individual clones was evaluated by G418 selection. 3 clones survived G418 to a significant degree, and the best of these clones, designated MCF7/ETR, showed G418 resistance in nearly 100% of cells after ecotropic virus infection. This clone was selected for further studies.

2. Characterization of tamoxifen and drug sensitivity in MCF7 cells

An unexpected logistic problem arose with obtaining an antiestrogen for this study. We have originally proposed to use 4-hydroxytamoxifen (4-OHTAM), a metabolite of tamoxifen which has been predominantly utilized in the literature for *in vitro* cytotoxicity studies (10). 4-OHTAM is not commercially available, and we have therefore requested a sample from a company (ICI), which in the past provided this reagent to researchers. We were eventually informed that this company no longer holds a license for 4-OHTAM, and we were directed to a French company as the new license holder. The latter company, however, did not reply to our request for this reagent. We have therefore decided to use tamoxifen itself, rather than 4-OHTAM, for our tissue culture work, The only disadvantage of tamoxifen relative to 4-OHTAM is that it requires approximately two orders of magnitude higher concentration for in vitro cytotoxicity (personal communication from Dr. Craig Jordan). Since tamoxifen is readily available from commercial sources, we have substituted 4-OHTAM with tamoxifen for all the relevant studies in this project.

We have analyzed the sensitivity of MCF7/ETR cells to tamoxifen, as well as doxorubicin (adriamycin), vincristine, cytarabine, and cisplatin, using a standard plating efficiency colony assay. Representative assays are shown in Figs. 1-5, where MCF7/ETR cells, transduced with an insert-free LNCX retroviral vector and selected with G418, were used as a control to study the effects of a BCL2-derived GSE (see below) on drug sensitivity. In these assays, cells were exposed to drugs for a short period of time (1.5-4 hrs, except for tamoxifen which required 24 hr treatment) and then allowed to form colonies for 5-8 days. These conditions mimic bolus administration, the most common form of therapy administration in the clinics, and reveal primarily the cytotoxic rather than the cytostatic effects of the drugs.

3. Chemosensitization of MCF7 cells by a GSE from the BCL2 gene

Active cell death (apoptosis) is a physiological process

which is primarily responsible for the cytotoxicity of many anticancer drugs (11). Apoptosis induced by many different stimuli is inhibited by the product of the BCL2 oncogene (12). Gene transfer and expression of BCL2 in BCL2-negative cells has been shown to confer resistance to different classes of anticancer drugs, indicating that BCL2 is a multidrug resistance gene (13,14).

Using the single-gene GSE selection strategy (3), we have previously isolated a single sense-oriented peptide-encoding fragment of BCL2 cDNA which acted as a GSE, sensitizing BCL2-expressing cells to drug induced apoptosis. The original selection had been carried out in a B-cell leukemia cell line. The isolated GSE, termed 2-7, and its variant, differing by a single amino acid and designated 2-25, were found to increase drug sensitivity in AA2 and HL60 leukemia and SW480 carcinoma cell line (15). We have now tested this GSE in MCF7 breast carcinoma cells, and found that the efficacy of the BCL2-derived GSE was the highest in this cell line. Fig. 1 shows that 2-7 sensititizes MCF7 cells to vincristine by approximately one order of magnitude. Significant sensitization of MCF7 cells by the BCL2-derived GSE was also observed with tamoxifen, doxorubicin, cytarabine and cisplatin (Figs. 2-5). The GSE, which encodes a short (28 amino acids) peptide from the middle of the BCL2 protein, was at least as efficient in MCF7 cells as a construct expressing full-length antisense RNA of BCL2, expressed from the same LNCX retroviral vector.

When investigating the effect of the 2-7 element on BCL2 gene expression, we have found to our surprise that this peptide-encoding GSE drastically reduces the steady-state level of BCL2 mRNA in MCF7 cells. This is illustrated by an experiment in Fig. 6, where BCL2 mRNA expression was analyzed by a RT-PCR assay (16), using the following BCL2-specific primers: GTGGCCTTCTTTGAGTTCGG (sense) and GGCTCAGATAGGCACCCAGG (antisense). Primers amplifying β_2-microglobulin cDNA (16) were used as an internal control. The mechanism of this striking effect is as yet unknown.

4. Isolation of GSEs inducing doxorubicin resistance in MCF7 cells

Doxorubicin (Adriamycin) is the most clinically efficient chemotherapeutic agent currently in use for breast cancer (1). To identify the genes responsible for doxorubicin sensitivity in breast carcinoma, we have used MCF7/ETR cells to carry out the selection of GSEs conferring resistance to doxorubicin, starting from a normalized random fragment cDNA library that we had constructed from human HeLa cells (5). The library, which contains about 2×10^7 recombinant clones, was introduced by retroviral transduction into 4×10^7 MCF7/ETR cells, on four P150 plates, which were then subjected to doxorubicin selection under the following protocol: 2.5 hrs exposure to 150 nM doxorubicin, followed by 2 days without drug, then another drug treatment under the same conditions, and the final round of treatment, this time increasing the drug dose to 500 nM. After this selection, only 6 surviving colonies in a with the control population, infected with the LNCX insert-free vector virus, but library-

containing cells survived at an average rate of 27 colonies per plate. Over 60 individual colonies were picked, expanded and frozen. Analysis of proviral inserts integrated in representative clones from this set by PCR (4) showed that most of the tested clones contained only single or a few inserts (Fig. 7). These results indicate that GSE selection is succesfully proceeding in this system.

5. Preparation of a population of normalized cDNA fragments from MCF-7 cells

The normalized cDNA library from HeLa cells, used for doxorubicin selection, is unlikely to be useful for tamoxifen selection, since genes responsible for antiestrogen sensitivity most probably are not expressed in HeLa cells. We are therefore constructing a normalized random fragment cDNA library from MCF7 breast carcinoma cells. Poly(A)$^+$ RNA was isolated from MCF7 cells and used to synthesize cDNA by reverse transcription using a random primer, conjugated at its 5' end with an adaptor that carries translation termination codons in all three open reading frames and the SalI site. Another adaptor, carrying three translation initiation codons and the SalI site, was ligated to both ends of the cDNA fragments; this adaptor will be subsequently removed from the 3' ends after ClaI digestion. The cDNA population prepared by this procedure would have different adaptors at the 5' and 3' ends, which will simplify subsequent orientation of the selected GSEs. The double-stranded cDNA fragments, ranging from 150 to 1000 bp in size, were successfully amplified by PCR using adaptor-specific primers (Fig. 8). We are currently optimizing the conditions for hydroxyapatite separation of single-stranded and double-stranded DNA, as a preliminary step to the normalization of our MCF7 cDNA preparation.

Conclusions

In the course of the above described studies, we have established that (i) the apoptosis suppressor, BCL2, plays a critical role in chemosensitivity of MCF7 breast carcinoma cells and thus presents a promising target for chemosensitization of breast cancer; (ii) a sense-oriented GSE from BCL2 decreases BCL2 mRNA levels in MCF7, indicating the existence of a previously unknown and potentially exploitable mechanism of BCL2 regulation; (iii) a normalized cDNA library from human HeLa cells is a suitable source for the isolation of GSEs that confer doxorubicin resistance in breast carcinoma cells. Other studies in this project are still at a stage which is too early to draw specific conclusions.

The originally proposed studies will be continued with the following changes:

(1) Tamoxifen instead of 4-OHTAM will be used to select for GSEs conferring antiestrogen resistance.

(2) We have originally planned to clone GSEs conferring chemotherapeutic drug resistance in other cell types and then test them on MCF7 cells. However, MCF7/ETR cells proved to be an appropriate recipient cell line to isolate GSEs conferring doxorubicin resistance from a HeLa-derived normalized cDNA

library, and therefore doxorubicin-resistance GSEs will be selected directly in MCF7/ETR cells.

(3) The striking chemosensitizing efffect of the BCL2-derived GSE in MCF7 cells warrants further analysis. We will therefore determine if the GSE indeed functions through peptide production and whether it affects the regulation of BCL2 mRNA stability, translation or protein modification. We will also seek additional funding to fully explore this exciting observation.

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Appendix

Figure Legends

Fig. 1. Effects of clone 2-7 (BCL2-derived GSE) and of a clone expressing antisense RNA of *BCL2* in the same LNCX vector on vincristine resistance of MCF7/ETR cells. Cells were treated with vincristine for 1.5 hrs and then cultured for 8 days. Cell survival (relative to untreated cells) was determined by a plating efficiency colony assay.

Fig. 2. Effects of clone 2-25 (a variant of 2-7 with a single amino acid change) and of a clone expressing antisense RNA of *BCL2* on tamoxifen resistance of MCF7/ETR cells. Cells were treated with tamoxifen for 24 hrs and then cultured for 8 days. Cell survival (relative to untreated cells) was determined by a plating efficiency colony assay.

Fig. 3. Effects of clone 2-7 and of a clone expressing antisense RNA of *BCL2* on doxorubicin resistance of MCF7/ETR cells. Cells were treated with doxorubicin for 2.5 hrs and then cultured for 5 days. Cell survival (relative to untreated cells) was determined by a plating efficiency colony assay.

Fig. 4. Effects of clone 2-7 and of a clone expressing antisense RNA of *BCL2* on cytarabine resistance of MCF7/ETR cells. Cells were treated with cytarabine for 1.5 hrs and then cultured for 8 days. Cell survival (relative to untreated cells) was determined by a plating efficiency colony assay.

Fig. 5. Effects of clone 2-7 and of a clone expressing antisense RNA of *BCL2* on cisplatin resistance of MCF7/ETR cells. Cells were treated with cisplatin for 4 hrs and then cultured for 8 days. Cell survival (relative to untreated cells) was determined by a plating efficiency colony assay.

Fig. 6. Effects of clones 2-7, 2-25 and antisense *BCL2* RNA on *BCL2* mRNA expression in MCF7/ETR cells. Analysis was carried out by RT-PCR, using β_2-microglobulin (B2M) as an internal control.

Fig. 7. Recovery of cDNA inserts from retroviral vectors integrated in the genomic DNA of doxorubicin-selected clones of MCF7/ETR cells. The inserts were amplified by PCR using primers derived from vector and adaptor sequence. M: 123 bp ladder. 1-16: individual cell clones.

Fig. 8. Electrophoretic analysis of the population of cDNA fragments from MCF7 cells, amplified by PCR using adaptor-derived primers. M1: "1 kb ladder" size standards (BRL). M2: 123 bp ladder. 1-3: cDNA fragments, amplified by PCR from 1:10, 1:100 and 1:1000 dilutions of the ligation mixture.

Sensitization of MCF-7 to vincristine by BCL2 GSE or full-length antisense RNA

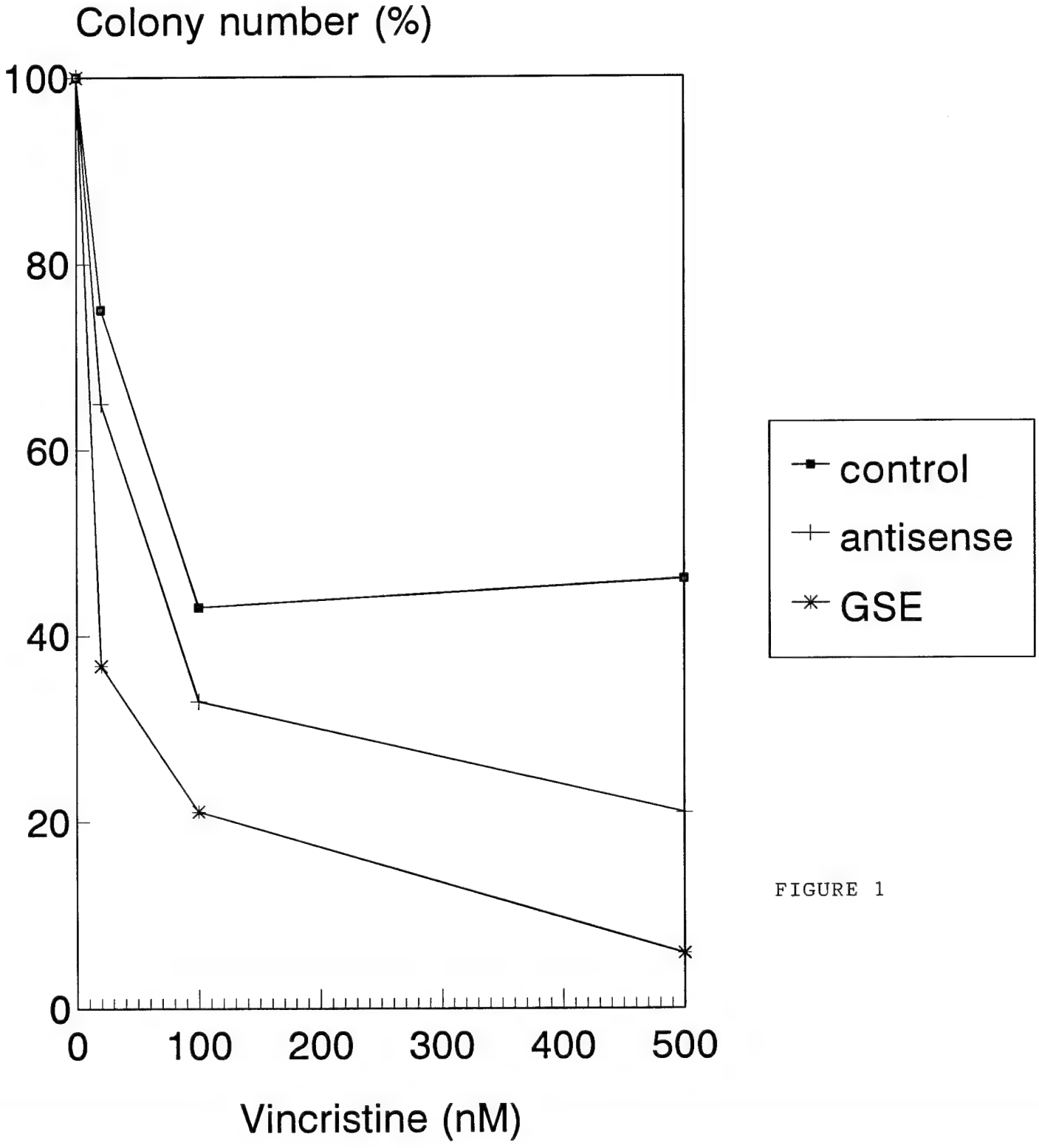

FIGURE 1

Sensitization of MCF-7 to tamoxifen by BCL2 GSE or full-length antisense RNA

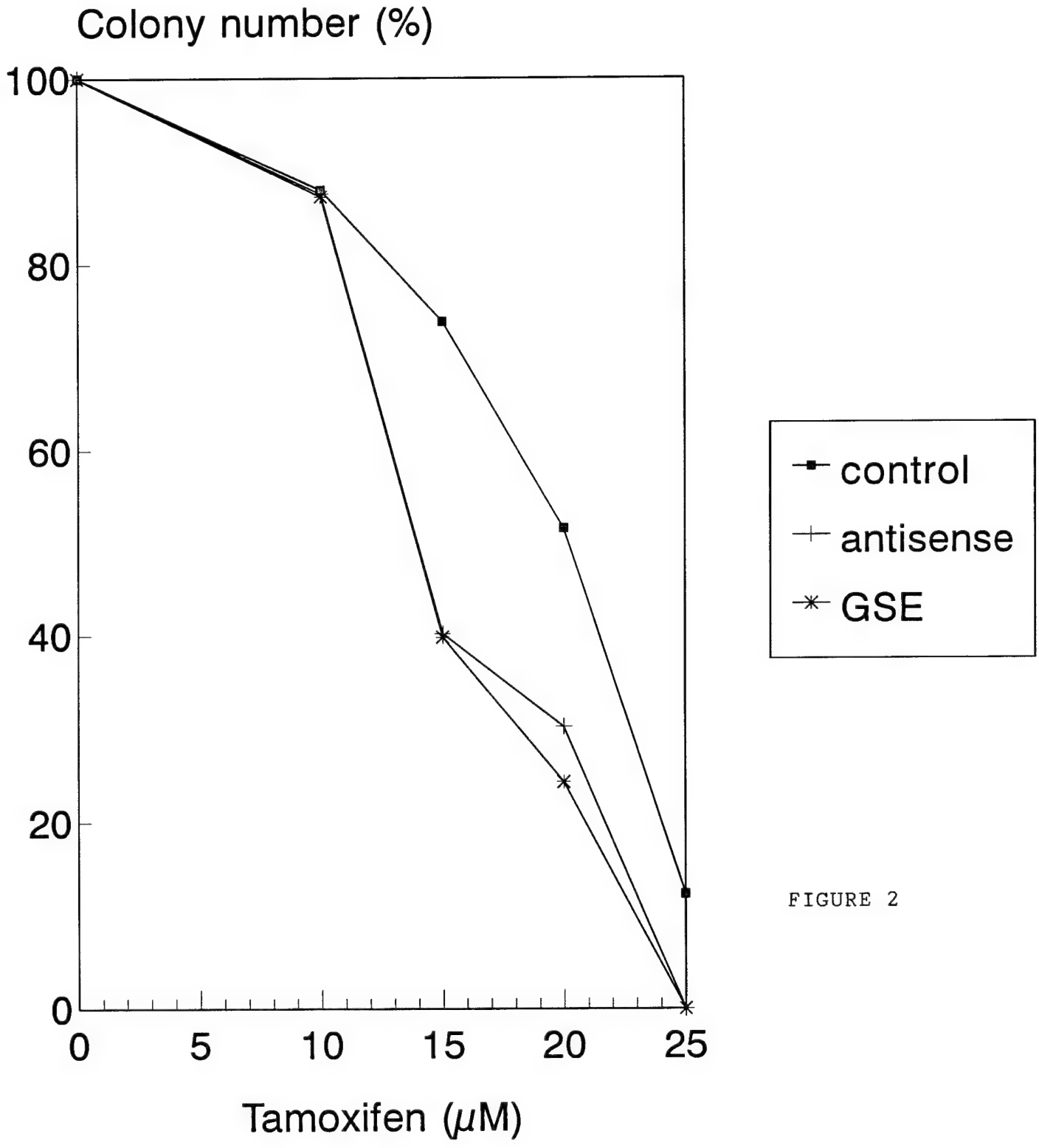

FIGURE 2

Sensitization of MCF-7 to doxorubicin by BCL2 GSE or full-length antisense RNA

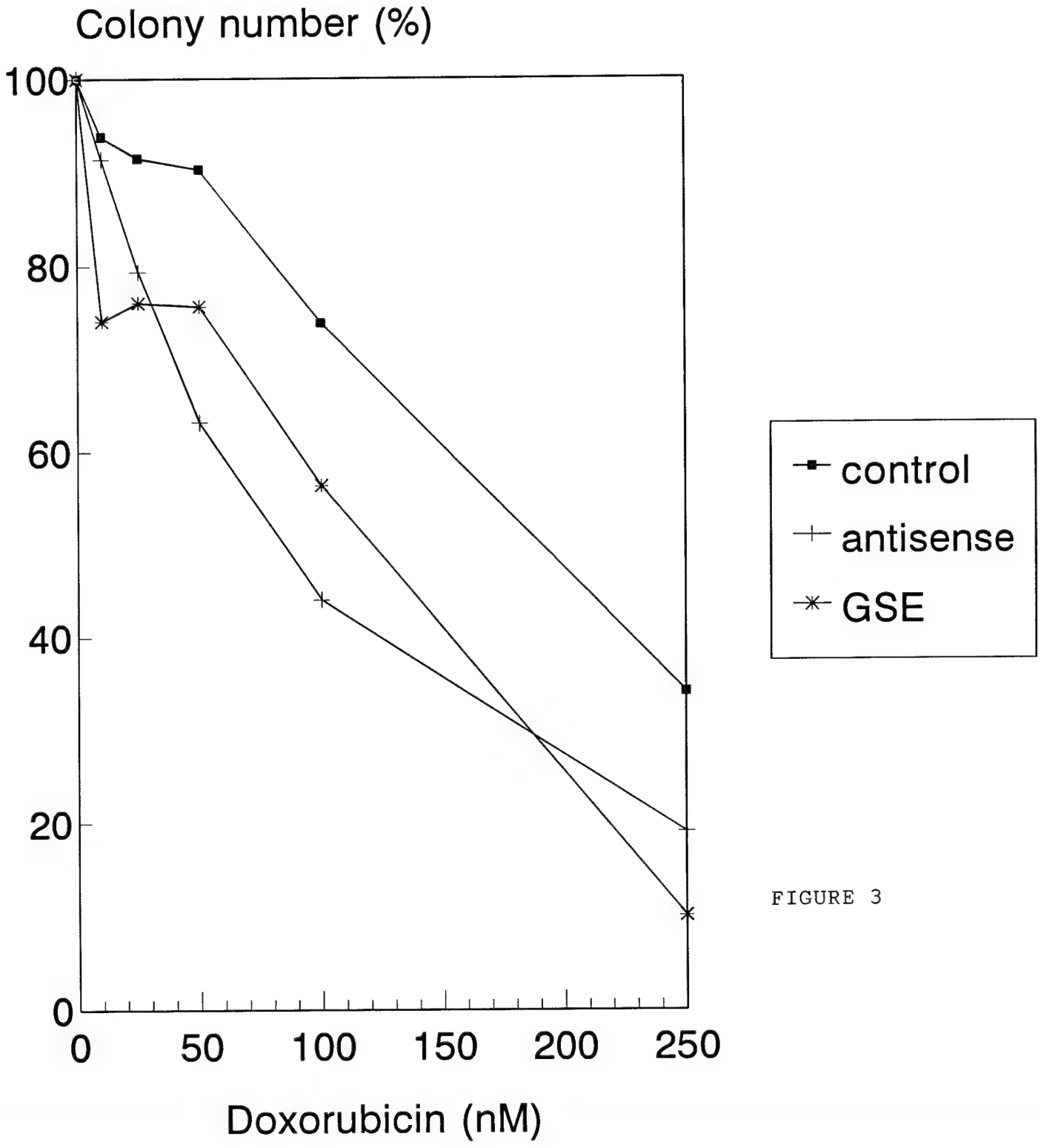

FIGURE 3

Sensitization of MCF-7 to cytarabine by BCL2 GSE or full-length antisense RNA

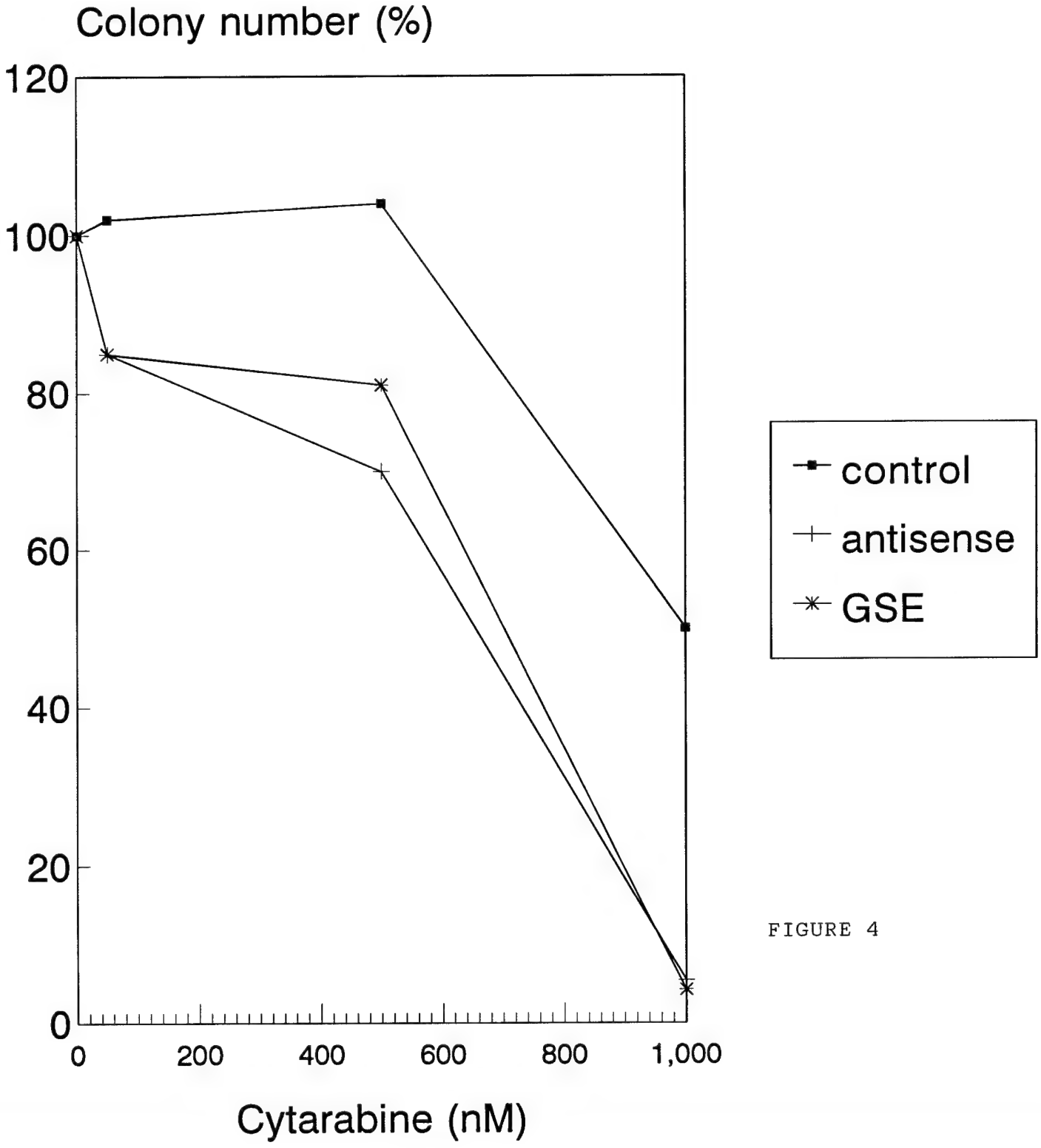

FIGURE 4

Sensitization of MCF-7 to cisplatin by BCL2 GSE or full-length antisense RNA

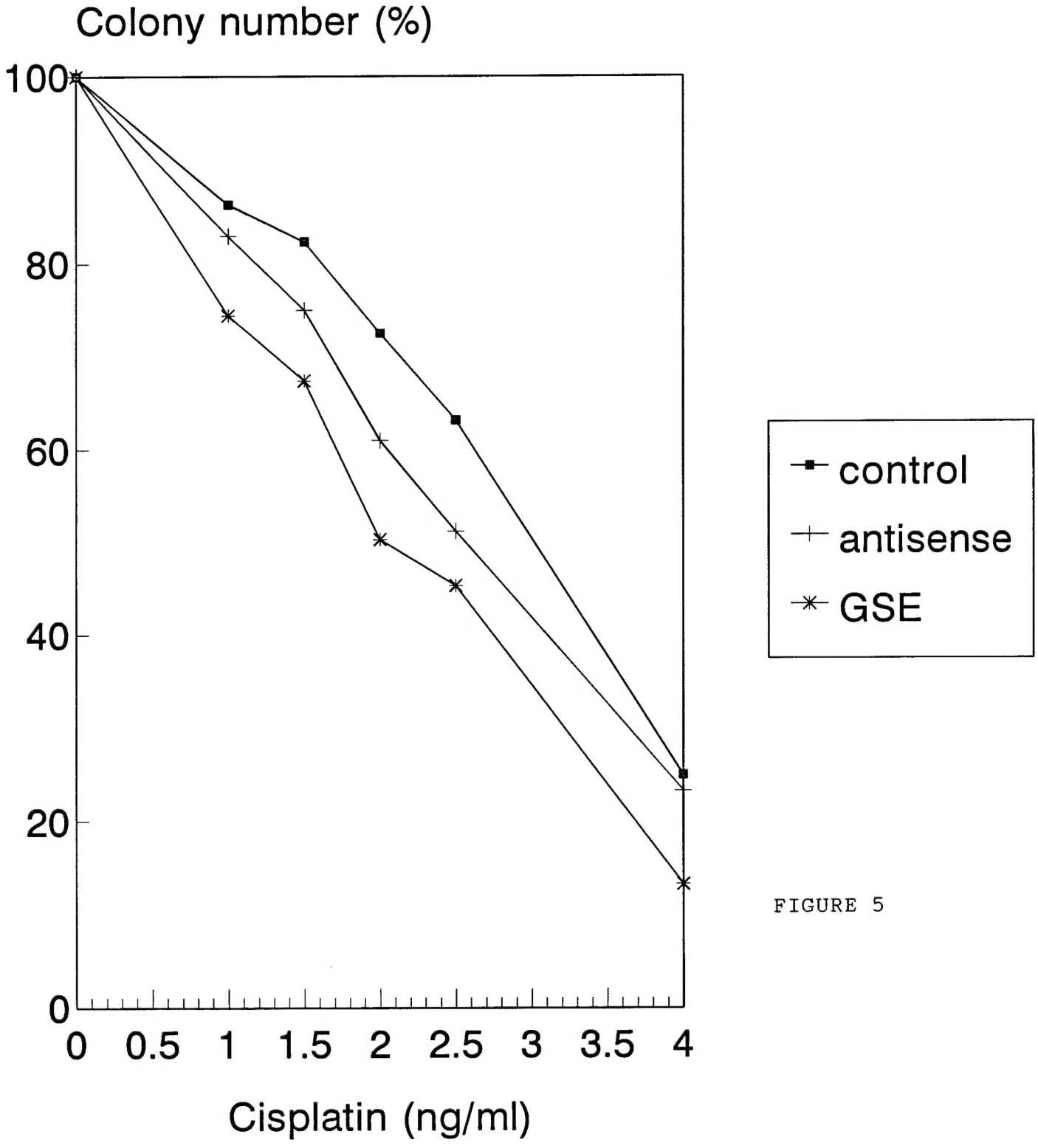

FIGURE 5

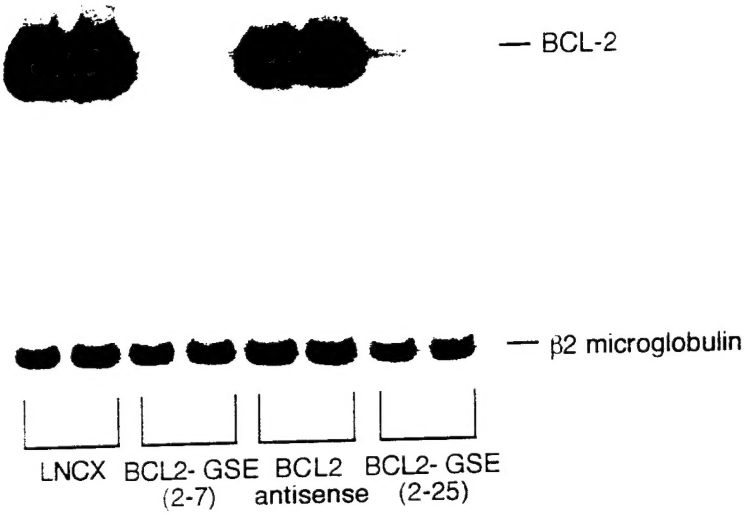
— BCL-2
— β2 microglobulin
LNCX
BCL2- GSE (2-7)
BCL2 antisense
BCL2- GSE (2-25)

FIGURE 6

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M

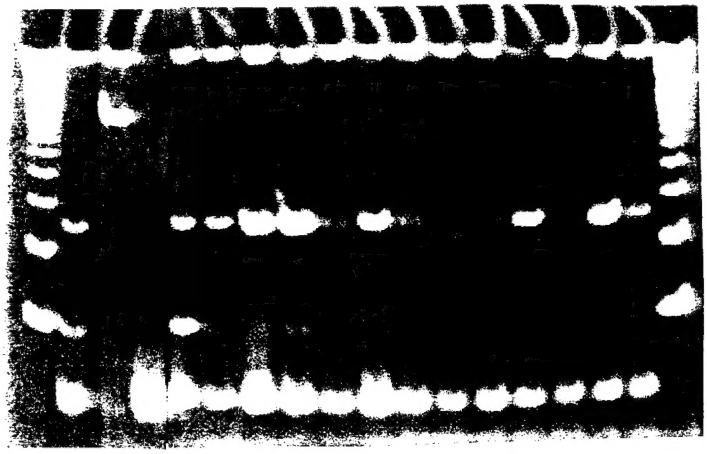

FIGURE 7

FIGURE 8